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FOREWORD

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INTRODUCTION

The long-term objective of this project is to develop a new treatment for breast cancer based on blockade of the autocrine growth factor activity of procathepsin D. Breast cancer cells secrete procathepsin D, the enzymatically inactive form from which the aspartic proteinase cathepsin D is generated by removal of an activation peptide (APpCD). Procathepsin D has been identified as an independent prognostic factor in several forms of cancer, particularly breast cancer. In preliminary experiments, procathepsin D was found to act as a specific autocrine growth factor for breast cancer-derived cells, but not for any other cell type tested. These effects were mediated through a specific receptor expressed on breast cancer cell lines that is distinct from the usually proposed cathepsin D-specific mannose-6-phosphate receptor. The region of procathepsin D responsible for its mitogenic activity was localized to APpCD and amino acids 27-44 of the APpCD sequence. No growth factor activity could be shown with the mature enzyme cathepsin D. The proposed specific aims are based on the central hypothesis that procathepsin D is involved in breast cancer via a specific receptor that mediates autocrine activation for increased metastatic growth. This project proposed the following aims: 1) it is hypothesized that the overproduction of procathepsin D results in an increase in the metastatic potential of breast tumor cells. A low metastatic human breast cancer cell line will be transfected with human procathepsin D cDNA such that the cells will secrete constitutively varying amounts of procathepsin D. The metastatic potential of each transfected cell line will be evaluated both *in vitro* and *in vivo* in relationship to the amount of procathepsin D secretion. 2) Attempts will be made to determine the exact site in procathepsin D responsible for breast cancer cell growth factor activity. Synthetic peptides representing fragments of APpCD will be prepared. Amino acid substitutions in the most active peptide fragment will be used to map the essential amino acid contact sites for the receptor. 3) It is hypothesized that inhibition of the APpCD interaction with its receptor will result in inhibition of cancer cell growth. Peptide analogs will be prepared with D-amino acids to block the growth and malignancy of cancer cells.

BODY

TASK 1

First set of MDA-MD-231 cell line transfected with various human procathepsin D cDNA are currently tested. Preliminary results shown in Figure 1 (see Appendices) suggested successful transfection. In addition, experiments establishing the basic values in invasiveness of parental cell line and the effect of procathepsin D have been established (see Figure 2, Appendices). Similarly, the effect of inhibition of procathepsin D secretion *in vivo* has been demonstrated (see Figure 3, Appendices). Based on these preliminary data, we believe that the **Task 1** will be successfully achieved in next future.

TASK 3

All necessary synthetic peptides and fragments were synthesized. After establishing that the fragment 27-44 is the part of the activation peptide responsible for the procathepsin D binding to the cancer cells (see Vetvicka et al., Breast Cancer Research and Treatment, 57: 261-269, 1999, Appendices), we focused our attention on this particular part of the activation peptide. We prepared the library of 10 individual peptides (see Figure 4, Appendices) which will be tested *in vitro*. The goal of these experiments is to pinpoint the exact binding moiety on the activation peptide molecule.

KEY RESEARCH ACCOMPLISHMENTS

- ❑ Demonstration that the binding site of the procathepsin D activation peptide is located in the 27 – 44 AA region
- ❑ Establishment of first cell lines transfected with human procathepsin D cDNA
- ❑ Establishment of basic values for invasiveness *in vitro* and *in vivo*
- ❑ Demonstration that invasiveness of breast cancer cells can be blocked by inhibition of procathepsin D secretion

REPORTABLE OUTCOMES

Abstracts:

1. Vetvicka,V., Vetvickova, J., Fusek, M.: Anti-procathepsin D activation peptide antibodies inhibit human breast cancer development, **VIIIth International Aspartic Proteinase Conference**, Funchal, Madeira, 1999, S-4-6
2. Vetvicka,V., Vetvickova, J., Fusek, M., Voburka, Z.: Blocking of growth factor activity of procathepsin D inhibits human cancer, *Faseb J.*, Abstracts, p. A1000, 2000.

CONCLUSIONS

Based on results mentioned above, we have all reasons to believe that this project will be finished in successful and timely manner. There is a slight delay in achieving all aims proposed in Task 1, mostly because some unforeseen problems with cDNA plasmids. However, all preliminary experiments establishing the basal levels of *both in vitro* and *in vivo* reactions have been established, therefore as soon as the trasnfected cells will be cloned and cultured in appropriate numbers, the rest of experiments will be done very quickly.

In addition, we managed to prepare and finish some of the experiments proposed for Tasks 2 and 3 (originally for months 15 – 26, and 16 – 36, resp.). In particular, two groups of mice are in process of immunization. The latests test revealed good level of immune response. We expect to perform the fusion for hybridoma approximately in June. As far as experiments in Task 3 are concerned, we already established that synthetic fragment corresponding to the 27-44 AA portion of activation peptide is responsible for binding of procathepsin D to the cancer cells. Subsequent experiments helped to locate the actual binding site to a region of 36-44 AA portion of the activation peptide.

Based on these results we proposed and purchased a library of synthetic peptides differing in a single amino acid (see Appendices). We are currently preparing the *in vitro* experiments for evaluation of the biological activity of these small synthetic fragments.

So far, all obtained data support the original hypothesis that procathepsin D significantly stimulates the growth and spreading of breast cancer cells. If this hypothesis is further confirmed by this research project, this project has very significant potential to be developed into preclinical trials leading toward a new, very specific treatment of human breast cancer. In addition, recent observation by our and other groups suggest that the role of procathepsin D in human cancer development is probably even more general than we originally believed. In such a case, this project might be even more important.

Appendices

Figure 1

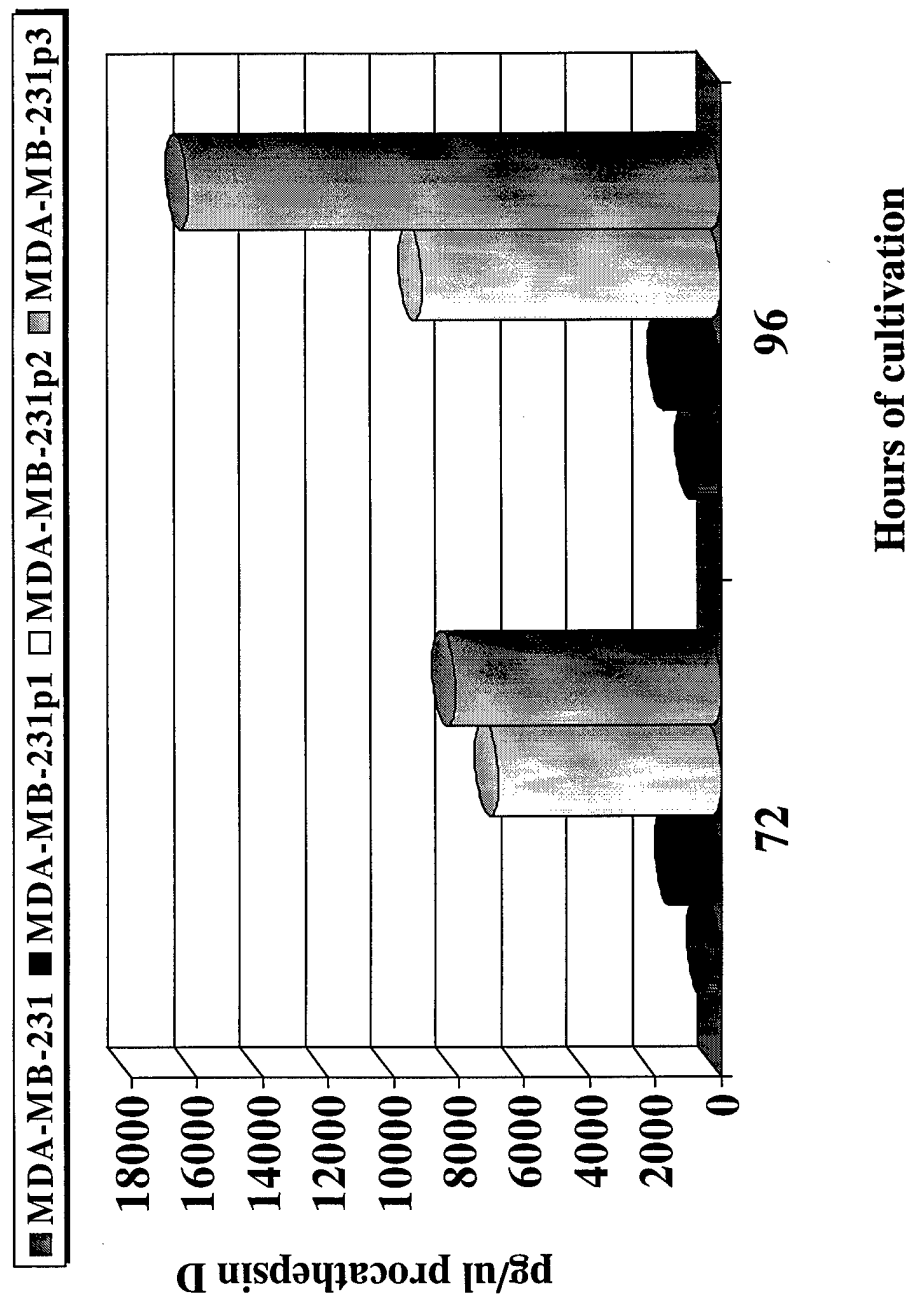


Figure 2

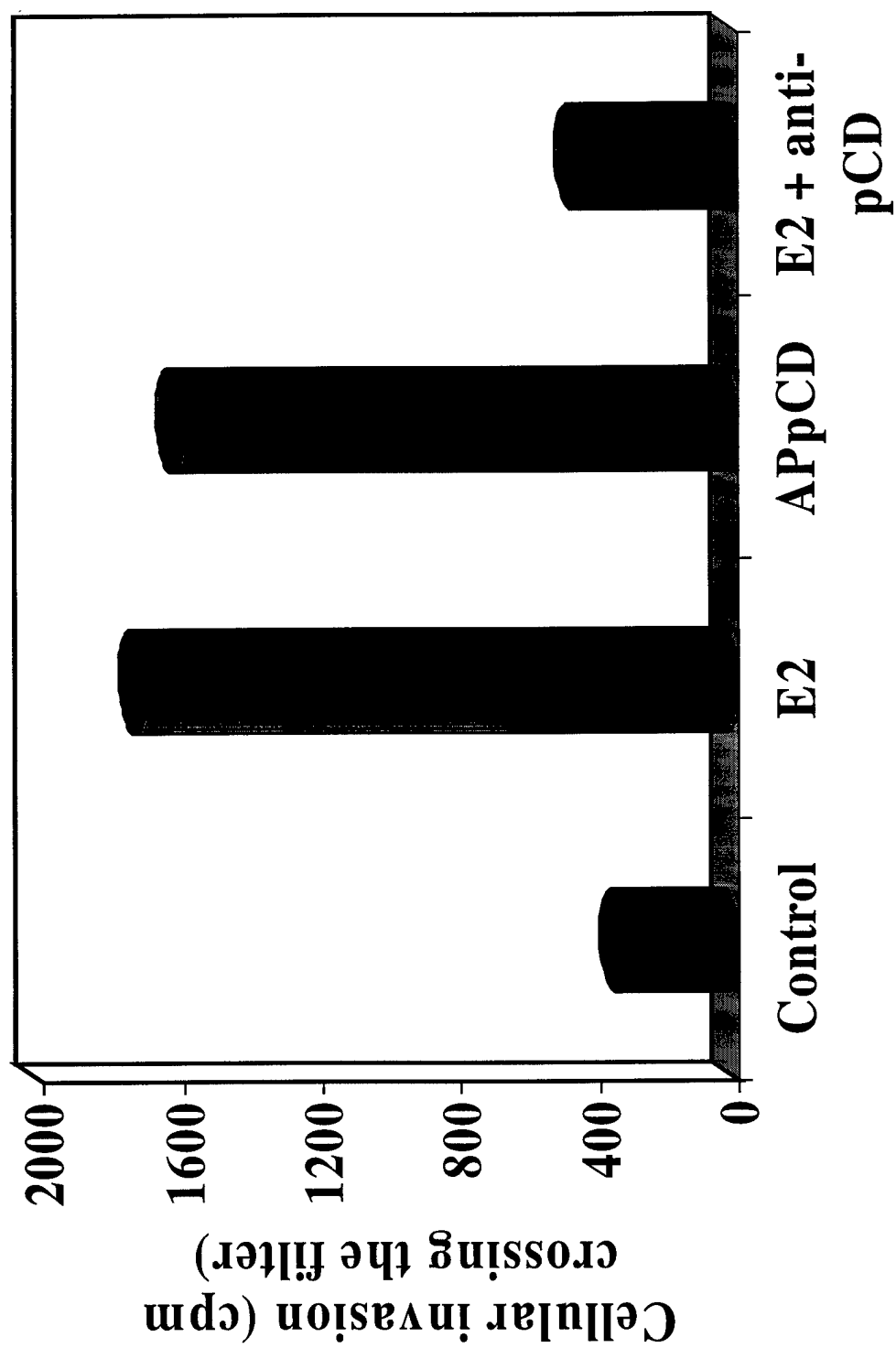
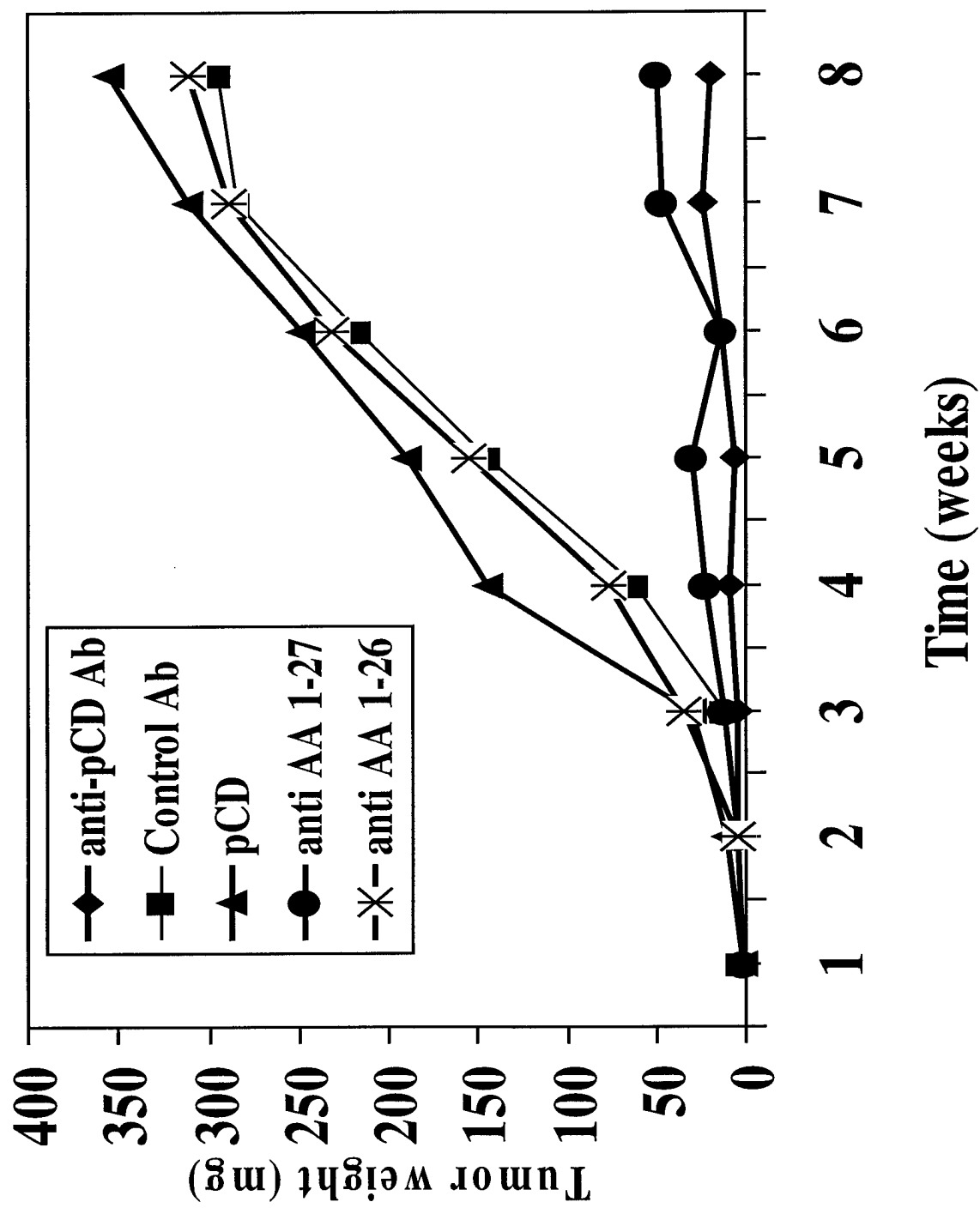


Figure 3



Legend to the Figures

Figure 1

Comparison of the procathepsin D secretion in tissue culture supernatants of three cell lines transfected with cathepsin D cDNA. Three different clones have been established. Using an ELISA assay employing anti-procathepsin D antibodies, we compared the levels of procathepsin D secreted into tissue culture supernatants of transfected and parental cells.

Figure 2

Inhibition of *in vitro* invasion by the anti-procathepsin D antibodies. Cells from serum-free cultures were tested for their ability to transmigrate a Matrigel membrane toward a 1% serum stimulus in the absence or presence of estradiol, procathepsin D and/or 100 ug/ml anti-procathepsin D antibodies.

Figure 3

Inhibition of breast cancer growth in athymic nude mice by treatment with anti-procathepsin D or anti-activation peptide fragment antibodies. A control antibody (anti-HLA) of the same isotype had no effect on tumor growth. Human tumors were generated by injecting athymic mice with 5×10^6 MDA-MB-231 cells directly into mammary fat pads. After two weeks, the mice were checked for tumor development. Mice with palpable breast tumors were then injected intravenously with biodegradable microspheres containing 100 ug of antibodies. Mice were sacrificed at various time intervals and tumor size was evaluated by weight. Mice treated with anti-procathepsin D or anti-fragment 27-44 AA antibodies showed greatly reduced tumor growth.



Report

Anti-human procathepsin D activation peptide antibodies inhibit breast cancer development

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Key words: activation peptide, antibodies, breast cancer, immunotherapy, procathepsin D

Summary

Enzymatically inactive procathepsin D secreted from cancer cells has been confirmed to play a role in development of human breast cancer. In the present study, we focused on the role of activation peptide which was in our preliminary studies suggested to be most probably responsible for mitogenic activity of procathepsin D. Using synthetic fragments and antibodies raised against individual fragments, we demonstrated that the growth factor activity of activation peptide is localized in a nine amino acid stretch (AA 36–44) of activation peptide and moreover both anti-activation peptide and anti-27–44 peptide antibodies administered *in vivo* inhibited the growth of human breast tumors in athymic nude mice.

Taking into account our previous results and presented data, we hypothesize that the interaction of procathepsin D activation peptide with an unknown surface receptor is mediated by a sequence 36–44 plus close vicinity. We also propose that this interaction leads in certain types of tumor derived cell lines to proliferation and higher motility. Blocking of the interaction of activation peptide by specific antibodies or antagonists might be a valuable tool in breast cancer inhibition.

Introduction

Involvement of human procathepsin D in cancer development has been confirmed over the past decade by several independent research teams [1, 2]. The prognostic value of procathepsin D tissue concentration was well documented in the case of node positive breast cancer. Much less is known about mechanisms of procathepsin D participation in cancerogenesis. Cell proliferation experiments demonstrated mitogenic properties of procathepsin D, and subsequently a long line of experiments was devoted to clarify the molecular basis for this growth promoting activity [3–5].

The extracellular mitogenic activity of procathepsin D was often interpreted solely as a consequence of proteolytic activity of cathepsin D [6, 7]. This approach neglects several facts, from which the most important seems to be that the inactive precursor, not the active enzyme, is over expressed and actively secreted.

This phenomenon has been documented many times [8, 9]. This over expression followed by extracellular release is in some cell lines controlled by estrogens, as has been proved on the DNA level [10–13]. Procathepsin D was shown to be one of the major secreted glycoproteins in the MCF-7 cell line [3]. However, the question of the estrogen effects on procathepsin D secretion is still not fully understood. Another important fact is that none of the previous experiments has shown mitogenic properties of added cathepsin D while addition of procathepsin D causes growth promotion, in particular, of cell lines [14].

We consider procathepsin D not simply as a precursor of enzymatically active proteinase but as a compact glycoprotein structure which possesses other functions than being only the zymogen of cathepsin D. In our earlier experiments we studied these molecular features and proposed that the most promising candidate for the mitogenic function is the activation peptide of procathepsin D molecule. Results of our

Table 1. Amino acid sequences of synthetic fragments of procathepsin D activation peptide

Peptide no.	Sequence	Numbering of the activation peptide sequence
656	IAKGPVSKYSQAVPAVTE II	27-44
657	TMSEVGGSVEDLIAKFPVSKYS	15-36
658A	SKYSQAVPAVTE II GPIPEVLKNY	33-10*
658V	SKYSQVVPAVTE II GPIPEVLKNY	33-10*

The position of first amino acid of mature cathepsin D is given by II.

*Amino acids of the mature enzyme.

experiments show that the structure most probably responsible for the mitogenic activity of extracellular procathepsin D is the activation peptide, which is part of the procathepsin D molecule until its proteolytic removal [9, 14, 15].

In the current report we present results which strongly indicate the importance of a structure within the activation peptide for mitogenic properties of procathepsin D. We were able to find a stretch of nine amino acids within the 44 amino acid long peptide which possessed practically the same properties as the whole procathepsin D molecule. The importance of these observations is pronounced especially by the facts that antibodies against synthetic activation peptide or its certain parts blocked binding of procathepsin D to cells [16], inhibited its mitogenic activity [14], and inhibited the growth of experimental breast cancer *in vivo* [9].

Materials and methods

Isolation of procathepsin D

Human procathepsin D was isolated from culture supernatants of human breast cancer cell line ZR-75-1 as described earlier [17]. Briefly, a two-step procedure was used. In the first step an immunoaffinity chromatography was used with anti-activation peptide antibodies attached to protein A Sepharose. In the second step FPLC chromatography using a Mono-Q column and 20 mM Tris (pH 7.2) were used.

Antibodies

Monoclonal antibodies were prepared in our laboratory against synthetic peptide sequences representing either the entire activation peptide or its fragments as described in Table 1. Each mAb was isolated from as-

cites fluid by 50% ammonium sulfate precipitation followed by Mono-Q anion exchange chromatography. All used antibodies were characterized as IgG₁ using Mouse MonoAb ID Kit (Zymed, San Francisco, CA). Monoclonal anti-human HLA-DR+DP IgG₁ antibody MEM-13 was a generous gift from Dr V. Horejsi (Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic). Another control antibody was MOPC-21 IgG purchased from Sigma (St. Louis, MO).

Cell cultivation

For growth experiments, cells were first incubated for 2 days in 0.1% FCS. The cells were harvested by centrifugation and washed six times in Iscove's modified Dulbecco's medium with HEPES buffer supplemented with glutamine, antibiotics, and 10 µg/ml of human transferrin [24]. Cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells per milliliter (150 µl per well) in the presence or absence of different concentrations of purified pCD or various substances tested in triplicate wells. After several days in culture, the proliferation was evaluated using an MTT assay [14]. In experiments with β -estradiol, cells were incubated in serum-free conditions with β -estradiol and with anti-APpCD or irrelevant monoclonal antibodies for 5 days. In all cell culture experiments, the cells were seeded in triplicate.

Synthetic peptides

The 44-amino-acid-long peptide corresponding to the activation peptide of pCD and the smaller fragments were synthesized in the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic. The purity of the APpCD and its fragments was controlled by using following methods: HPLC, amino acid analysis.

and mass spectrometry. The HPLC method showed the purity to be more than 95%. The amino acid analysis confirmed the amino acid composition. Mass spectrometry results were in accordance with the proposed molecular structure. In addition to techniques mentioned above, the purity of peptides was controlled also by N-terminal sequencing using automated system where the first nine N-terminal amino acids were in agreement with the designed structure. The synthetic 40 AA peptide corresponding to the intracellular part of the human CR3 receptor with almost identical molecular weight as APpCD was used in some experiments as a control.

Chemicals

RPMI 1640 medium, β -estradiol, HEPES, MTT, and transferrin were obtained from Sigma (St. Louis, MO), fetal calf serum was from Hyclone Laboratories (Logan, UT), protein A Sepharose from Pharmacia (Pharmacia LKB Biotechnology, Piscataway, NJ), and pig pepsinogen A (EC 3.4.23.1) from Worthington (Freehold, NJ).

Animals

Athymic nu/nu BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Human cell lines

The MCF-7 cell line was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). Human breast cancer cell lines ZR-75-1 and MDA-MB-231 were obtained from Dr. R. Ceriani of the John Muir Cancer and Aging Research Institute, Walnut Creek, CA. The cancer cell lines were grown in RPMI 1640 medium with HEPES buffer supplemented with 10% heat-inactivated PCS, 2 mM L-glutamine, 100 units per milliliter penicillin, and 100 μ g/ml streptomycin in plastic disposable tissue culture flasks at 37°C in a 5% CO₂/95% air incubator.

Preparation of biodegradable microspheres

Biodegradable gelatin microspheres were prepared as described in [18].

Tumor cell growth in mice

Human breast tumors were generated as described previously [9] by injecting athymic nude mice with

5×10^5 MDA-MB-231 cells directly into mammary fat pads. After 10–14 days, the mice were checked for tumor development and only mice with palpable tumors were subsequently injected intravenously with biodegradable gelatin microspheres containing 100 μ g of either anti-APpCD mAb, anti-fragment mAbs, or irrelevant anti-human MEM-13 IgG₁ anti-HLA-DR+DP mAb (two injections 4 weeks apart). Mice were sacrificed at various time intervals and tumor size was evaluated by weighing the tumors. Four mice were used in each time interval.

Results

Results showing the specificity of the activation peptide-mediated proliferation of human breast cancer cells are shown in Figure 1. These results are comparable to previously published data showing the growth factor effects of procathepsin D exclusively on breast cancer cells, but not on six other types of tumor-derived cell lines [2–4]. Mature enzyme cathepsin D had no effects in our system (data not shown). Figure 2 summarizes the time curve of the activation peptide-potentiated growth of breast cancer cells. In all three tested cell lines we found a linear growth suggesting the doubling time being approximately 24 h. Based on these results, we used a five-day incubation in all subsequent experiments. When the breast cancer cells are cultivated *in vitro* in serum free conditions without

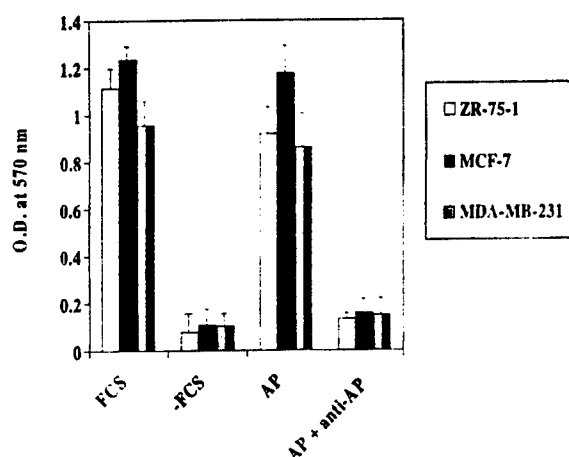


Figure 1. Growth of human breast cancer cell lines in serum-free medium containing 50 ng/ml of activation peptide (AP) \pm 1 μ g/ml of anti-activation peptide monoclonal antibody (anti-AP). The results from growth in the serum-free medium (-FCS) and in control medium supplemented with FCS (+FCS) are given for comparison. The results represent the mean \pm SD of three independent experiments.

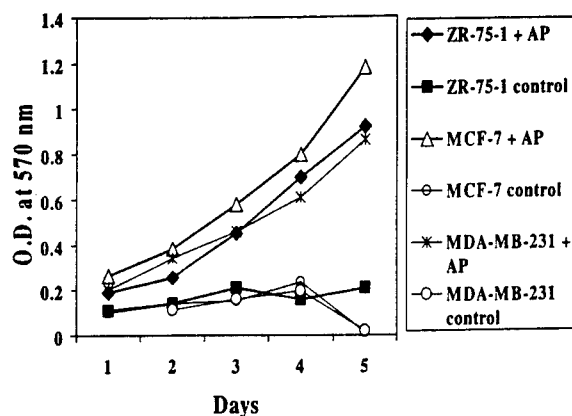


Figure 2. Time curve of the activation peptide (50 ng/ml) potentiated growth of three human breast cancer cell lines in serum-free medium. The results from growth in the control serum-free medium without the activation peptide (control) are given for comparison. The results are representative of three independent experiments.

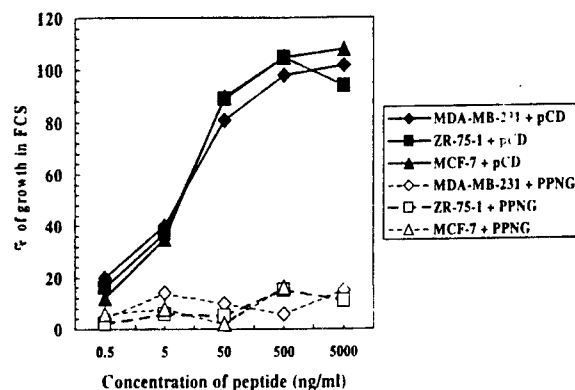


Figure 3. Dose-dependent proliferative activity of procathepsin D with three human breast cancer cell lines in serum-free medium. The results represent the mean of three independent experiments.

addition of any growth factor, they do not proliferate and usually start to die around day 5 of cultivation.

The effects of various concentrations of either procathepsin D or its activation peptide are shown in Figures 3 and 4. When growth factor activity of the activation peptide was evaluated, an irrelevant synthetic peptide with similar purity and molecular weight representing the intracellular part of the human CR3 receptor was used. Pig pepsinogen A was used as a control for evaluation of growth factor activity of the whole procathepsin D. Pepsinogen is a protein of similar overall structure. Previous studies showed that mature enzyme cathepsin D or bovine cathepsin D are not active under similar experimental conditions [11].

The synthetic peptides representing fragments of the procathepsin D activation peptide are summarized

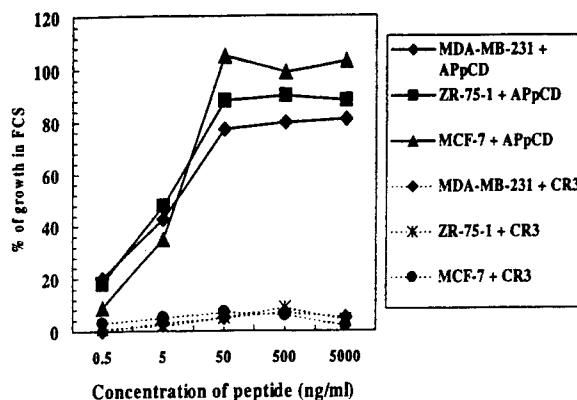


Figure 4. Dose-dependent proliferative activity of an activation peptide (APpCD) with three human breast cancer cell lines in serum-free medium. The proliferation of cell lines in dependence on the control peptide (see Material and methods section for further details) are given for comparison. The results represent the mean of three independent experiments.

in Table 1. Our previous observations demonstrated that the growth factor activity of activation peptide is localized in the second 18 (AA 27-44) amino acids [9]. Based on our knowledge of the three-dimensional structure of active cathepsin D [19], we prepared peptide 657 (AA 15-36) with only part of the active fragment, and two peptides, 658A and 658V (AA 33-10) having 10 amino acids of the mature enzyme cathepsin D. In addition, these two peptides differ in one amino acid substitution, 658V peptide having valine6 instead of alanine6. This valine6 mutation is potentially significant due to Rochefort's finding that this polymorphism is common in cathepsin D isolated from human tumors [21]. The authors suggested that this missense polymorphism might modify procathepsin D secretion and or maturation in breast cancer cells. We compared the mitogenic activity of these peptides with the peptide 656 (AA 27-44).

In the following set of experiments we measured the growth-stimulating effects of individual fragments of activation peptide (Figures 5 and 6). In each experiment we compared the possible inhibitory effects of anti-fragment antibodies to antibody against activation peptide. These data also demonstrated the specificity of action of individual fragments. The control antibody MOPC-21 IgG showed no blocking effects (data not shown). The cultivation conditions were identical to those used in Figure 1, that is, the cells were cultivated in serum-free conditions.

In simultaneous experiments we evaluated the effects of anti-activation peptide antibodies on β -estradiol-induced proliferation of breast cancer cell

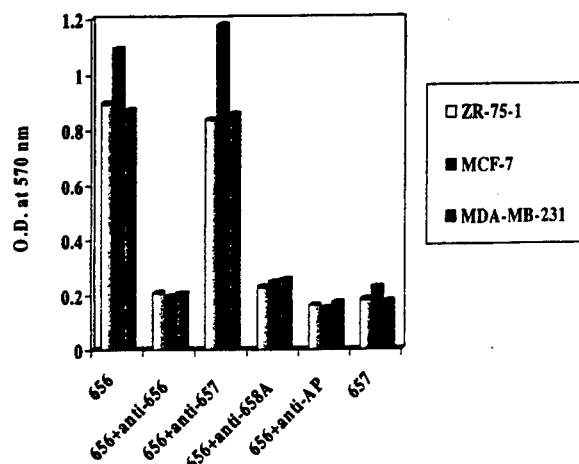


Figure 5. Stimulation of breast cancer cell growth by fragment 656 and its inhibition by various anti-fragment monoclonal antibodies (1 μ g/ml). Fragment 657 was added as control. The results represent the mean of three independent experiments.

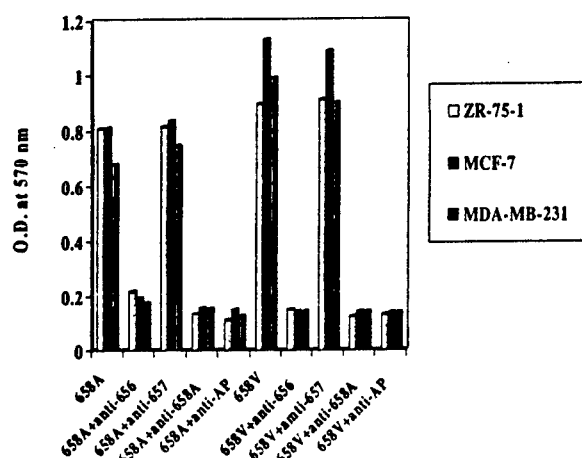


Figure 6. Stimulation of breast cancer cell growth by fragments 658A and 658V and its inhibition by various anti-fragment monoclonal antibodies (1 μ g/ml). The results represent the mean of three independent experiments.

lines growing in serum-free conditions. We found that both β -estradiol and activation peptide stimulate growth of ER⁺ cell line ZR-75-1, but the activation peptide-induced stimulation occurs with approximately one day delay (Figure 7). Simultaneous addition of anti-activation peptide antibody to either activation peptide or β -estradiol inhibited the cell proliferation. When anti-activation peptide antibody was used alone, no proliferation was observed (data not shown). These data were confirmed in subsequent experiments where we found a significant inhibition of growth of the same cell line regardless of the used concentration

of β -estradiol (Figure 8). The same concentration of anti-activation peptide IgG (1 μ g/ml) was used in all our experiments. And again, when anti-activation peptide antibody was used alone, no proliferation was observed (data not shown).

The previously mentioned experiments were followed by a final set of experiments where we focused on the growth-stimulating activity of procathepsin D in an *in vivo* model in which both anti-activation peptide and anti-656 antibodies were shown to reverse breast cancer development. Human breast cancer cell line MDA-MB-231 cells (which lack the estrogen receptor) were injected directly into mammary fat pads of the athymic nude mice. After evaluation for development of tumor mass, mice were injected with biodegradable gelatin microspheres containing 100 μ g of antibodies. Figure 9 shows that mice treated with either anti-656 or anti-procathepsin D antibodies had greatly reduced tumor growth. On the other hand, irrelevant anti-HLA antibodies or anti-657 antibodies (657 fragment did not cause specific growth of breast cancer cell lines) had no effect on cancer growth. The antibody-mediated protection from an induced breast cancer was almost complete for the whole tested interval of 8 weeks.

Discussion

In this paper, we focused on the hypothesis that the mitogenic growth factor activity of procathepsin D is mediated by its activation peptide [9]. This possibility is generally supported by several functions which the activation peptide was found to play within the family of aspartic proteinases. For example, yeast proteinase A uses structure within its activation peptide for targeting to the vacuole [15]. In the case of procathepsin D it is also speculated that a structure within the activation peptide is involved in targeting to lysosomes (in addition to classical mannose-6-phosphate pathways). As the direct mitogenic action has been already demonstrated [4, 14, 16], the aim here is to determine which amino acid residues in the activation peptide are responsible for receptor activation. Using synthetic peptides mimicking parts of the activation peptides and monoclonal antibodies against these peptides, we tested the properties of these molecules in both *in vitro* and *in vivo* conditions. Similarly, several laboratories including ours reported that in addition to the classical pathway of binding to the mannose-6-phosphate receptor [22], pCD binding is mediated

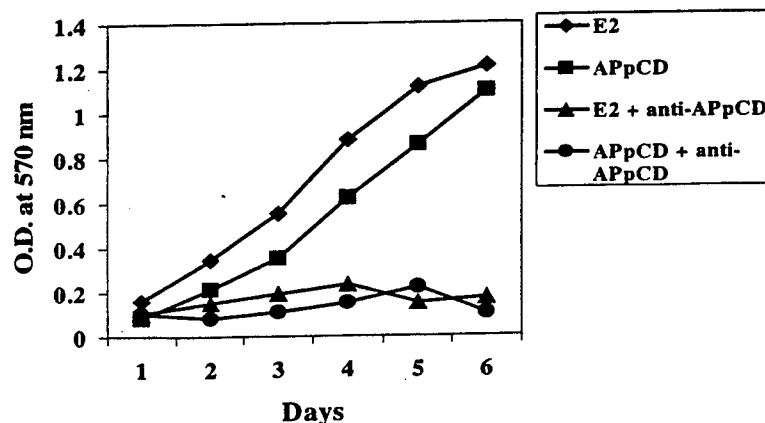


Figure 7. Both β -estradiol (E2) and activation peptide (APpCD) stimulated proliferation of breast cancer cell line ZR-75-1 can be inhibited by addition of anti-activation peptide (anti-APpCD) antibodies (1 μ g/ml). The results represent the mean of three independent experiments.

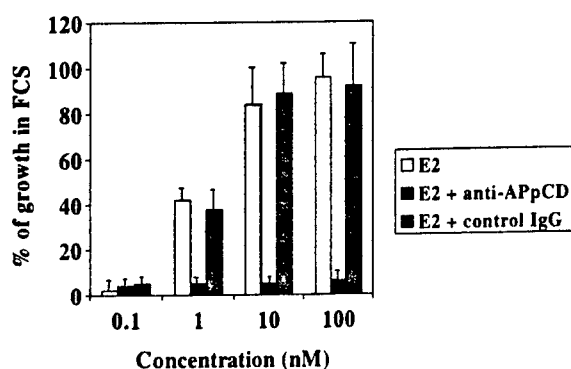


Figure 8. Inhibition of β -estradiol-stimulated growth of breast cancer cells by anti-activation peptide (1 μ g/ml) is not dependent on concentration of β -estradiol. Irrelevant anti-HLA antibodies of the same isotype were used as control. The results represent the mean \pm SD of three independent experiments. The cells were incubated in presence or absence of relevant molecules and the proliferation was evaluated at day 5 of incubation.

both by mannose-6-phosphate independent binding [23] and by another unidentified polypeptide [9, 24, 25].

Preliminary experiments have demonstrated that most and possibly all of the mitogenic activity of procathepsin D resides in amino acids 27-44 of the activation peptide sequence, and that no activity is found in amino acids 1-26 [9]. Based on the three-dimensional structure of cathepsin D and the suggested model of procathepsin D [19, 20], five synthetic peptides representing the entire activation peptide sequence and various fragments of it were synthesized, purified (Table 1), and compared for their mitogenic activity. Peptide 657 was constructed to assess the possibility that the active site responsible for mitogenic

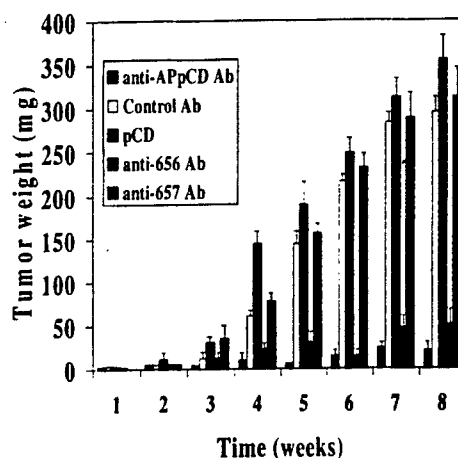


Figure 9. Inhibition of breast cancer growth in athymic mice by treatment with anti-activation peptide (anti-APpCD Ab) or anti-656 antibodies. Control antibody of the same isotype (anti-HLA) or anti-657 antibody had no effect on cell growth. See Material and methods for details. Results represent mean of four mice per time interval.

activity surrounds positions 25-27, and thus splitting of the entire activation peptide into peptides 1-26 and 27-44 would make them less effective. The substitution of Ala (peptide 658A) for Val in peptide 658V corresponds to the missense polymorphism found by Rochefort's group to be frequent in human tumor-derived cathepsin D [21]. Rochefort hypothesizes that this polymorphism might be involved in a number of factors contributing to the role of procathepsin D in breast cancer, including the secretion, mitogenic activity, and processing of procathepsin D.

To explore further the role of these peptidic sequences, monoclonal antibodies recognizing epitopes

corresponding to each of the peptide sequences were generated and tested for their ability to block growth factor activity *in vitro*.

In the first set of experiments we picked the optimal concentrations and time interval of our experiments. Based on finding that the growth of cells reached maximum around day 5 of incubation, we used this day as an endpoint of all subsequent experiments. These data were in agreement with our previous findings using intact procathepsin D on different breast cancer cell lines [9]. Throughout the study we used the addition of entire activation peptide (AA 1-44) as a positive control. Based on results of several groups, only breast cancer cells (all six tested cell lines) and three prostate cancer cells are sensitive to the addition of procathepsin D [3, 4, 9, 26]. Human B lymphoblastoid cells (Raji), T lymphoblastoid cells (8402), monocytoid cells (U937), epitheloid carcinoma cells (HELA), hepatocellular carcinoma cells HBL-100, or angiosarcoma cells (HAEND-1) were not responsive [4].

Next, the same experimental design was used for testing of synthetic fragments of procathepsin D activation peptide. Several conclusions can be made from our data summarized in Figures 5 and 6. First, they clearly demonstrate the specificity of our assay, as the antibodies raised against individual fragments always blocked the activity of the particular peptide. Second, several antibodies managed to inhibit the action of other peptides with partly overlapping sequence. Growth factor activity of peptide 656 (AA 27-44) is inhibited by peptide 658 (AA 33-10), activity of peptide 658 is blocked by 656, but not by 657 (AA 15-36), and peptide 657 is not active at all. Based on the results, we can predict that the binding moiety responsible for binding of activation peptide to the cancer cells is located somewhere around amino acids 36-44. However, the possibility that the anti-657 antibody reacts with the epitope which excludes amino acids 33-36 cannot be ruled out.

Finally, we checked directly the possible involvement of procathepsin D in breast cancer progression *in vitro*. All our experiments published earlier excluded any effects of full mature enzyme cathepsin D on proliferation of breast cancer cells. Besides soluble mannose-6-phosphate receptors, we used anti-cathepsin D monoclonal antibodies and specific inhibitor pepstatin A. In all these control experiments we found no involvement of the mature enzyme [11]. Some previous reports, however, suggested the role of cathepsin D in extracellular matrix degradation [6,

7], it was therefore necessary to distinguish between the effects caused either by procathepsin D or cathepsin D. To be sure that we really measure the effects of procathepsin D and not the enzymatically active cathepsin D, we used activation peptide only. *In vitro* potentiation of cancer cell proliferation by procathepsin D, activation peptide, and β -estradiol was almost identical. Based on these data it was not possible to distinguish whether this stimulation was mediated by β -estradiol or by procathepsin D which was secreted as a result of β -estradiol addition. However, pre-incubation of cells in the simultaneous presence of β -estradiol and anti-activation peptide antibodies inhibited the proliferation of cancer cells, demonstrating that the responsible molecule is, in fact, procathepsin D actively released after β -estradiol treatment [10, 26].

The last set of experiments was dedicated to the effects of procathepsin D and its fragments *in vivo*. Athymic nude mice with established human breast tumors were injected twice with biodegradable synthetic microspheres containing various monoclonal antibodies. From our results we can conclude that the presence of either anti-activation peptide or anti-656 antibodies (raised against proliferation-stimulating AA 27-44 fragment) sufficiently protected nu/nu mice for up to 8 weeks, which was the entire interval of this experiment. At that time the control animals together with mice treated with irrelevant antibody or anti-657 antibody became moribund and the experiment had to be terminated. These results are in agreement with our older data demonstrating *in vivo* inhibition of breast cancer growth by anti-procathepsin D antibodies [9].

Our previously published data shows that the interaction of procathepsin D with breast cancer cells is not mediated by mannose-6-phosphate receptor, but by a new, previously unknown surface receptor. Originally we found the lack of inhibition by excess of mannose, but these data proved only that the binding of procathepsin D to the cancer cells is not mediated by carbohydrate recognition. The possibility that mannose-6-phosphate receptor might possess other non-carbohydrate recognition properties similar to some lectins could not be overlooked. Similarly to growth inhibition studies, we also found little inhibition of procathepsin D binding by competing fluid-phase mannose-6-phosphate receptor or various anti-mannose-6-phosphate receptor antibodies [9]. From these experiments we can conclude that mannose 6-phosphate receptor has only a minor role

in binding of procathepsin D to breast cancer cells. We also demonstrated that proteolytic activity of mature cathepsin D is not the mechanism of mitogenic function of procathepsin D under our experimental condition.

Taking into account our previous results and the presented experiments, we propose that the interaction of procathepsin D activation peptide with an unknown surface receptor is mediated by the sequence 36-44 plus close vicinity. We also propose that this interaction leads in certain types of tumor derived cell lines to a potentiated growth and higher motility. Blocking of the interaction of activation peptide by specific antibodies or analogs functioning as antagonists might be a valuable tool in breast cancer inhibition.

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